2-Methylvalerate formation in mitochondria of Ascaris suum and its relationship to anaerobic energy generation

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Summary. Anaerobic incubation of intact Ascaris suum mitochondria with pyruvate and propionate results in the formation of acetate, 2-methylvalerate, and 2-methylpentenoate and involves a rotenone sensitive, electron-transport associated phosphorylation. Malate inhibits 2-methylvalerate formation in these incubations, apparently by dissipating reducing power necessary for 2-methylvalerate formation. Indeed, malonate, an inhibitor of NADH-dependent fumarate reduction, dramatically stimulates 2-methylvalerate formation in incubations containing malate/propionate and malate/pyruvate/propionate but not pyruvate/propionate. In addition, malonate stimulates both 2-methylbutyrate and 2-methylvalerate formation in incubations with malate alone. The results of the present study suggest that branched-chain fatty acid synthesis in A. suum mitochondria is energy linked and that the inability of isolated, intact mitochondria to form branched-chain fatty acids from malate, their presumed physiological substrate, may result from an imbalance in the initial malate dismutation.

Introduction

The parasitic nematode, Ascaris suum, was the first organism in which a functional, energy-generating, anaerobic mitochondrion was described (Kmetec and Bueding 1961; Saz 1971, 1981). It has served as a model for the elucidation of similar anaerobic pathways in other parasitic helminths (Barrett 1981), many invertebrates (Hochachka and Mustafa 1972), and even ischemic rat heart muscle (Cascarano et al. 1976). In contrast to typical aerobic mitochondria, the anaerobic organelles present within body wall muscle of adult A. suum lack a tricarboxylic acid cycle and their electron-transport system is antimycin A and cyanide insensitive. In addition, any oxygen uptake results in the formation of hydrogen peroxide (Saz 1971; Ward and Fairbairn 1970). Unsaturated organic acids are used instead of oxygen as terminal electron acceptors and succinate and a number of reduced volatile acids, such as acetate, propionate, 2-methylbutyrate (2-MB), and 2-methylvalerate (2-MV), accumulate as end products of carbohydrate fermentation (Fig. 1; Saz 1981). The branched-chain fatty acids (BFA), 2-MB and 2-MV, arise from the con-

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Abbreviations: BFA branched-chain fatty acid; 2-MB 2-methylbutyrate; 2-MC 2-methylcrotonate; 2-MP 2-methylpentenoate; 2-MV 2-methylvalerate

Fig. 1. A Summary of anaerobic malate metabolism in A. suum mitochondria; AC acetate; FUM fumarate; MAL malate; PROP propionate; SUCC succinate; 2-MB 2-methylbutyrate; 2-MC 2-methylcrotonate; 2-MP 2-methylpentenoate; 2-MV 2-methylvalerate. B Pathway of electron-transport in A. suum; ECR enoyl CoA reductase; FR fumarate reductase; NADH-DH NADH-dehydrogenase; RQ rhodoquinone
densation and subsequent reduction of acetyl CoA and propionyl CoA. The five carbon 2-MB is formed by the condensation of an acetate unit with a propionate unit, while the six carbon, 2-MV is formed by the condensation of two propionate units (Saz and Weil 1960, 1962).

Studies with intact *A. suum* muscle strips and enzyme localization experiments have indicated that malate is the primary mitochondrial substrate (Saz 1971). Incubation of *A. suum* mitochondria with malate results in the accumulation of succinate and pyruvate, a Site I electron-transport associated phosphorylation, and ATP formed/malate consumed ratios of 0.5 (Kohler and Bachman 1980; Saz 1971). However, the branched-chain fatty acids, 2-MB and 2-MV, major end products of carbohydrate metabolism in intact muscle strips, are not found. This occurs in spite of the fact that *A. suum* muscle mitochondria do contain all of the enzymes necessary for BFA synthesis and do synthesize BFA when disrupted organelles are incubated in acetyl CoA, propionyl CoA and NADH (Komuniecki et al. 1981a, b; 1983; Suarez de Mata et al. 1977). Therefore, the present study was designed to examine further the inability of isolated, intact *A. suum* mitochondria to form BFA from malate and, more importantly, to assess the role of anaerobic electron-transport and possible energy generation in the pathway of BFA formation.

**Materials and methods**

*Chemicals.* CoA esters were obtained from P and L Biochemicals (Milwaukee, WI), 2-Methylcrotonate, 2-methyl-2-pentenoate and 2-methylbutyrate were obtained from Aldrich Chemicals (Milwaukee, WI) and 2-methylvalerate from Pfaltz and Bauer, Inc. (Stamford, CT). Liquifluor and Aquasol were obtained from New England Nuclear (Boston, MA). All other chemicals were of reagent grade and purchased from Sigma Chemical Company (St. Louis, MO).

*Tissue isolation and anaerobic incubation.* *Ascaris suum* body wall muscle strips were obtained by dissection within 6 hours of helminth isolation and mitochondria were isolated as described previously (Komuniecki et al. 1981a; Komuniecki and Saz 1979). The washed mitochondrial pellet was resuspended in 20 mM potassium phosphate buffer (pH 7.4), containing 200 mM sucrose, 1 mM MgCl2, and 10 mM KCl with one pass of a Teflon homogenizer. Incubations were conducted in duplicate at 30 °C in shaking Warburg respirometers after flushing with nitrogen for 10 min. The final incubation mixture contained 175 mM sucrose, 20 mM potassium phosphate (pH 7.4), 10 mM KCl, 15 mM glucose, 0.4 mM ADP, 1 mM MgCl2, 5 or 10 mM substrates (pH 7.4) and additions, as indicated in the text, in a final volume of 2 ml. Rotenone was added in 5 μl of ethanol with 5 μl of ethanol added to the other incubations as a control. The reaction was terminated by placing the incubation mixtures in a boiling water bath for 5 min. Then, the samples were neutralized and stored at –20 °C. Prior to analysis for volatile acids, the CoA esters were hydrolyzed by raising the pH to 11.0 with 1 N KOH and incubating at 70 °C for 20 min.

**Anaerobic phosphorylation.** Anaerobic phosphorylation was measured in standard Warburg vessels at 30 °C, as described above. Intact mitochondria (4–6 mg protein) were preincubated under N2 for 10 min with shaking in 120 mM sucrose, containing 20 mM Tris-HCl (pH 7.4), 25 mM glucose, 10 mM KCl, 0.4 mM ADP, 1 mM MgCl2, 10 mM 32P, potassium phosphate (pH 7.4; 2–6 x 105 cpm/μmol), and effectors, as indicated, in a final volume of 2 ml. The reaction was initiated by the addition of the substrate to the side arm and terminated after 30 min by the addition of 0.2 ml of 6% perchloric acid. The reaction mixtures were centrifuged at 12,000 x g for 20 min. Then, the neutralized supernatant fractions were treated with molybdic acid and 32P-organic phosphate was extracted with isobutanol and ether as described previously (Saz 1971). 32P-glucose remaining in the extracted aqueous phase was determined by liquid scintillation spectrometry using 10 ml of Liquifluor as the scintillation medium.

**Determination of volatile fatty acids.** Volatile fatty acids were isolated by two different methods. Frozen mitochondrial incubation media were thawed and, in the first method, were acid steam distilled (Saz and Weil 1960). After neutralization of the distillate, the salts were concentrated to dryness, as described previously (Komuniecki et al. 1981a). The dry salts were acidified with 60% phosphoric acid and immediately applied to a gas-liquid chromatographic column according to Annison (1954). In the second method, volatile acids were extracted with ether from acidified mitochondrial media (Rizzo 1980). Incubation media (2 ml) were centrifuged at 10,000 x g for 10 min and 70 μl of 2 N HCl was added to the supernatant fraction, followed immediately by the addition of 1 ml ether. Ether extraction was repeated, combined with the first extract and concentrated to 0.2 ml. Samples were analyzed by chromatography at 130 °C on a 10% FFAP/1% H3PO4 Chromosorb WAW 100/120 column in a Hewlett Packard 5790 gas-liquid chromatograph equipped with a 339A Integrator. Volatile acids were identified by their relative retention times, as compared to standards, and quantified by calculation of their peak heights. At the conclusion of the initial incubations, 1 μmole (0.1 ml) of isobutyrate was added as an internal control and final results were corrected to 100% isobutyrate recovery. Recoveries from acid steam distillation varied between 70 and 80%, while recoveries of propionate, 2-MB, 2-MV, 2-methylcrotonate and 2-methylpentenoate from ether extraction were greater than 90%. However, acetate recoveries by ether extraction were less than 60% and variable. Therefore, to quantify acetate with ether extraction, trace amounts of 14C-acetate were added to the media prior to recovery. 14C-Acetate eluted from the column was trapped in 0.05 M KOH and an aliquot assayed for radioactivity in Aquasol by scintillation spectrometry.

**Results**

**Coupling of 2-methylvalerate formation to an anaerobic, electron-transport associated phosphorylation**

Incubation of intact *A. suum* mitochondria with their physiological substrate, malate, results primarily in the formation of pyruvate and succinate and not the BFA characteristic of their fermentative metabolism (Kohler and Bachman 1980; Saz